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14. ABSTRACT

Genetic studies in the PI's laboratory demonstrated that mutation of the staphylococcal accessory regulator (*sarA*) limits biofilm formation in *Staphylococcus aureus* to a degree that can be correlated with increased antibiotic susceptibility and an improved therapeutic outcome in biofilm-associated infections. The goal of this project was to take therapeutic advantage of this observation by identifying small molecule inhibitors of *sarA* expression and/or function. To this end, we proposed two sets of experiments, the first being to carry out a large scale screen to identify inhibitors that offer the most promise. This was done using genetic reporter constructs proven to accurately reflect the functional status of *sarA*. The second was to then evaluate the therapeutic efficacy of the most promising inhibitors using established animal models of biofilm-associated infection. In our screen of >30,000 compounds, we identified 31 of potential interest, but secondary screens, including those assessing biofilm formation and the production of SarA itself, led us to focus on a single compound (ST028355). However, this compound was ultimately found to be unstable, thus greatly delaying and ultimately preventing our ability to carry out the intended *in vivo* studies. To overcome this, we began a collaboration with Dr. Peter Crooks, a medicinal chemist and Chairman of the Department of Pharmaceutical Sciences at the University of Arkansas for Medical Sciences. This led to the synthesis of over 200 analogs of this compound. The hope was to screen these analogs, as well as an additional library of compounds that is already available in the Crook's laboratory and consists of compounds with favorable pharmacological properties, to both optimize ST028355 and identify additional compounds of interest, thereby putting us in a position to employ structure activity relationship (SAR) studies to proceed to *in vivo* studies an ultimately to fully exploit the proposed therapeutic approach of targeting *sarA*. While we were unable to pursue the *in vivo* studies proposed in the original application, we did make tremendous progress towards our experimental objectives, and this progress forms the basis for a recently submitted application for a PRORP Expansion Award program.

15. SUBJECT TERMS
Staphylococcus, biofilm, staphylococcal accessory regulator, *sarA*, inhibitor

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Biofilm-associated infections, including those associated with bone and indwelling orthopaedic devices, pose a unique and devastating clinical problem (Cierny, 2011). The genesis of our PRORP proposal (OR090571: *sarA* as a target for the prevention and treatment of staphylococcal biofilm associated infection) was our demonstration that mutation of the staphylococcal accessory regulator (*sarA*) limits biofilm formation in diverse strains of *Staphylococcus aureus* to a degree that can be correlated with increased antibiotic susceptibility (Beenken et al., 2003, Weiss et al., 2009a, Weiss et al., 2009b). Subsequent studies not only confirmed this phenotype but also demonstrated that mutation of *sarA* imposes a greater limitation on biofilm formation than any other *S. aureus* regulatory locus we have examined (Atwood et al., in press). Moreover, we demonstrated that mutation of *sarA* also limits the pathological consequences of staphylococcal bacteremia and the development of hematogenous bone infection (Blevins et al., 2002, Blevins et al., 2003, Zielinska et al., 2012) as well as the bone destruction that characterizes post-traumatic osteomyelitis (unpublished observations). These observations emphasize the therapeutic potential of identifying small molecule inhibitors of *sarA*-mediated regulation, and this was the overall focus of our research effort.

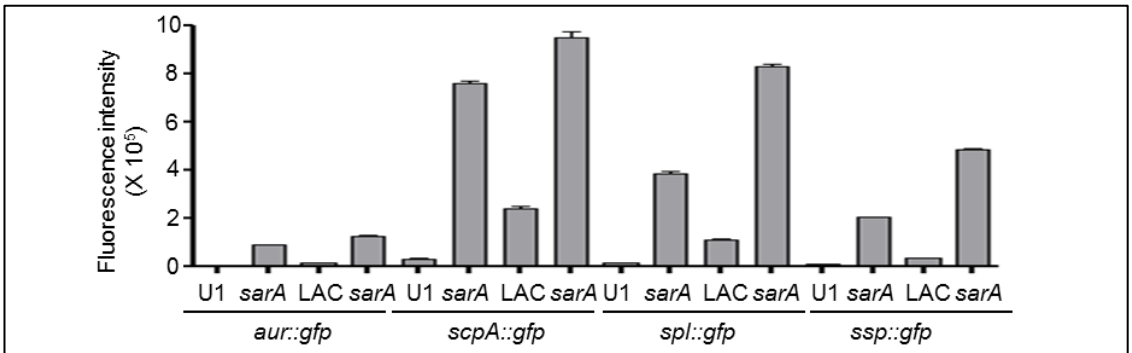

Fig. 1. Fluorescence with *gfp* reporters. The promoter regions from the iindicated protease genes/operons were fused to the gene encoding superfolder GFP (sGFP). The resulting constructs were introduced into the *S. aureus* strains UAMS-1 (U1), LAC, and their isogenic *sarA* mutants and the relative levels of fluorescence assessed after 4 hrs.

This effort was complicated by the global regulatory roles played by *sarA* (Cassat et al., 2006, Morrison et al., 2012) and the fact that screening for inhibitors based on the production of SarA itself does not lend itself to high throughput assays. Thus, we proposed to take advantage of our studies confirming that mutation of *sarA* results in a dramatic increase in the production of extracellular proteases and that this accounts for all of the clinically relevant phenotypes cited above (Beenken et al., 2010, Tsang et al. 2008, Zielinska et al., 2012). To this end, we generated green fluorescent protein (*gfp*) reporters to the promoters of all four *S. aureus* protease genes/operons and confirmed that fluorescence was in fact enhanced in *sarA* mutants with all four reporters relative to the fluorescence levels observed in the isogenic parent strains (Fig. 1). Using a luciferase (*lux*) reporter of one of these (*sspA::lux*), we then developed a high-throughput screen based on increased bioluminescence in a wild-type strain in the presence of the test compound, thereby allowing us to carry out our screen using a 96-well microtiter plate format (Fig. 2). Using this assay, we explored a number of avenues to identify inhibitors of *sarA* expression and/or function (Hobby et al., 2012, Ordonoz et al., 2011, Quave et al., 2012), some of which took advantage of previous studies investigating the impact of extracts from plants on *S. aureus* biology (Quave et al., 2008), while others focused on more targeted methods aimed at disrupting the interaction between SarA molecules as a means of preventing formation of a functional dimer (Xu et al., 2006). However, our primary effort was focused on screening a commercially available (TimTec)

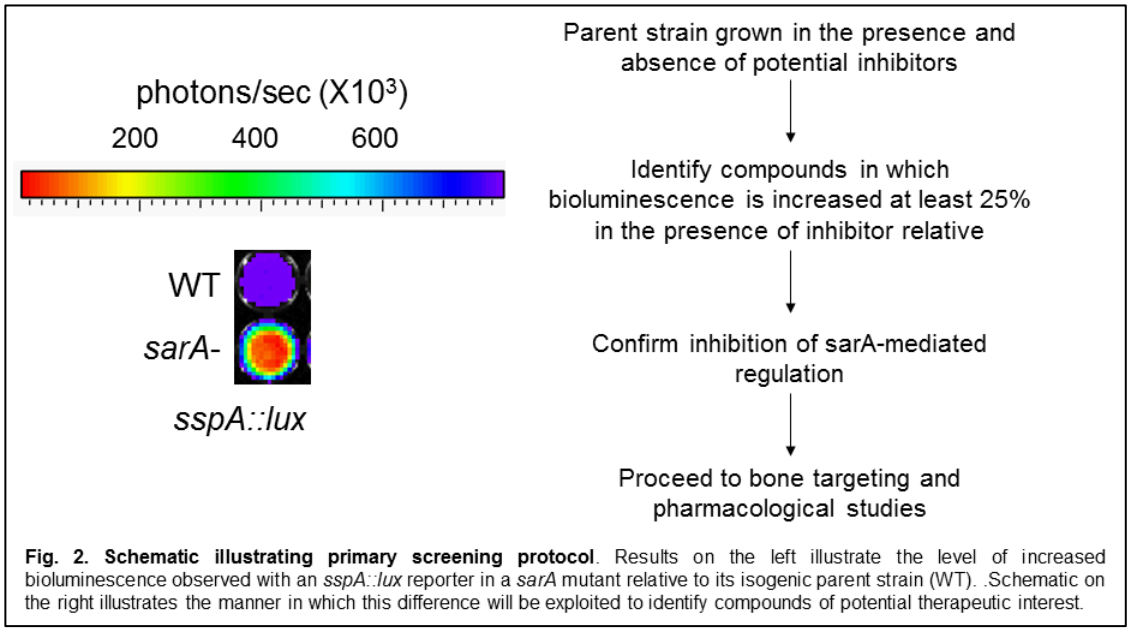

Fig. 2. Schematic illustrating primary screening protocol. Results on the left illustrate the level of increased bioluminescence observed with an *sspA::lux* reporter in a *sarA* mutant relative to its isogenic parent strain (WT). .Schematic on the right illustrates the manner in which this difference will be exploited to identify compounds of potential therapeutic interest.

small molecule library. To this end, we screened over 30,000 compounds and identified 31 that resulted in increased bioluminescence (see previous Progress Report). We then focused on the 3 most active of these compounds, but 2 were problematic owing to solubility issues. Thus, we focused our efforts of the 3rd compound (Compound 28, TimTec ST028355), which was subsequently shown to limit biofilm formation in a dose dependent manner to a degree comparable to mutation of *sarA* (Fig. 3) and, as assessed by western blot, to limit the accumulation of SarA itself (Fig. 4).

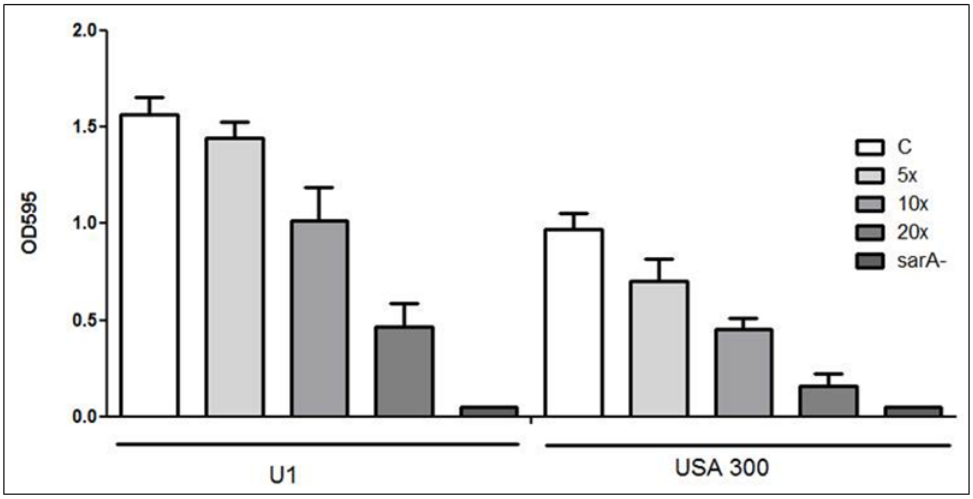

Fig. 3. Dose and strain dependent activity of ST028355. Results shown demonstrate the dose dependent inhibition of biofilm formation in the methicillin-sensitive strain UAMS-1 (U1) and the methicillin-resistant USA300 strain LAC. 5, 10 and 20X refer to increasing concentrations of the compound relative to that used in the primary screen. C refers to control results observed in the absence of any compound. The isogenic *sarA* mutant of each strain was included as an additional control.

This primary screen was done using very limited amounts of each compound kindly provided by Dr. Paul Dunman at the University of Rochester Medical Center. We obtained additional Compound 28 from TimTec, but unfortunately we were unable to reproduce these promising results. Subsequent analysis by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy confirmed that the structure of the compound we were provided was not consistent with the reported structure (data not shown), and based on we initiated a collaboration with Dr. Peter A. Crooks, Ph.D., D.Sc. and Chair of the Pharmaceutical Sciences Department at the University of Arkansas for Medical Sciences, who has a distinguished history in drug discovery and development and was in fact recruited to UAMS specifically to promote such efforts. Indeed, Dr. Crooks is the author of over 1,000 papers that focus on the development of therapeutic compounds in many different clinical contexts. Specifically, the Crook's laboratory carried out a *de novo* synthesis of this compound. This newly synthesized compound did increase bioluminescence with our *sspA::lux* reporter, but it was also found to be unstable. Thus, a key component of our efforts moving forward was to address this issue by synthesizing more stable and potentially more active derivatives of Compound 28, and we have successfully synthesized ~200 such compounds (Fig. 5). In addition, we had hoped to expand our screen to include a well-defined library of ~3,000 compounds, all of which have been developed by the Crooks' laboratory, and all of which have been shown to be non-toxic to mammalian cells and have drug-like properties favorable for clinical development, but unfortunately we were unable to screen any of these compounds within the context of our PRORP grant. We have, however,

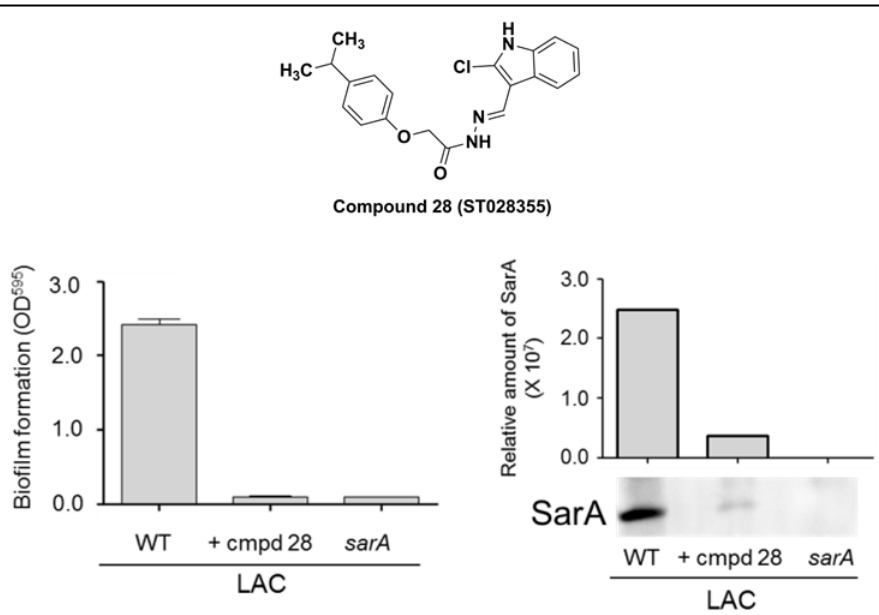

Fig. 4. Structure and biological activity of Compound 28. The structure of Compound 28 is shown along with assays demonstrating its impact on biofilm formation (left) and the accumulation of SarA as assessed by western blot (right). Impact of mutating *sarA* in the USA300 strain LAC is also shown for comparison.

submitted a proposal as part of the PRORP Expansion Award program that is pending review and will hopefully allow us to complete these studies.

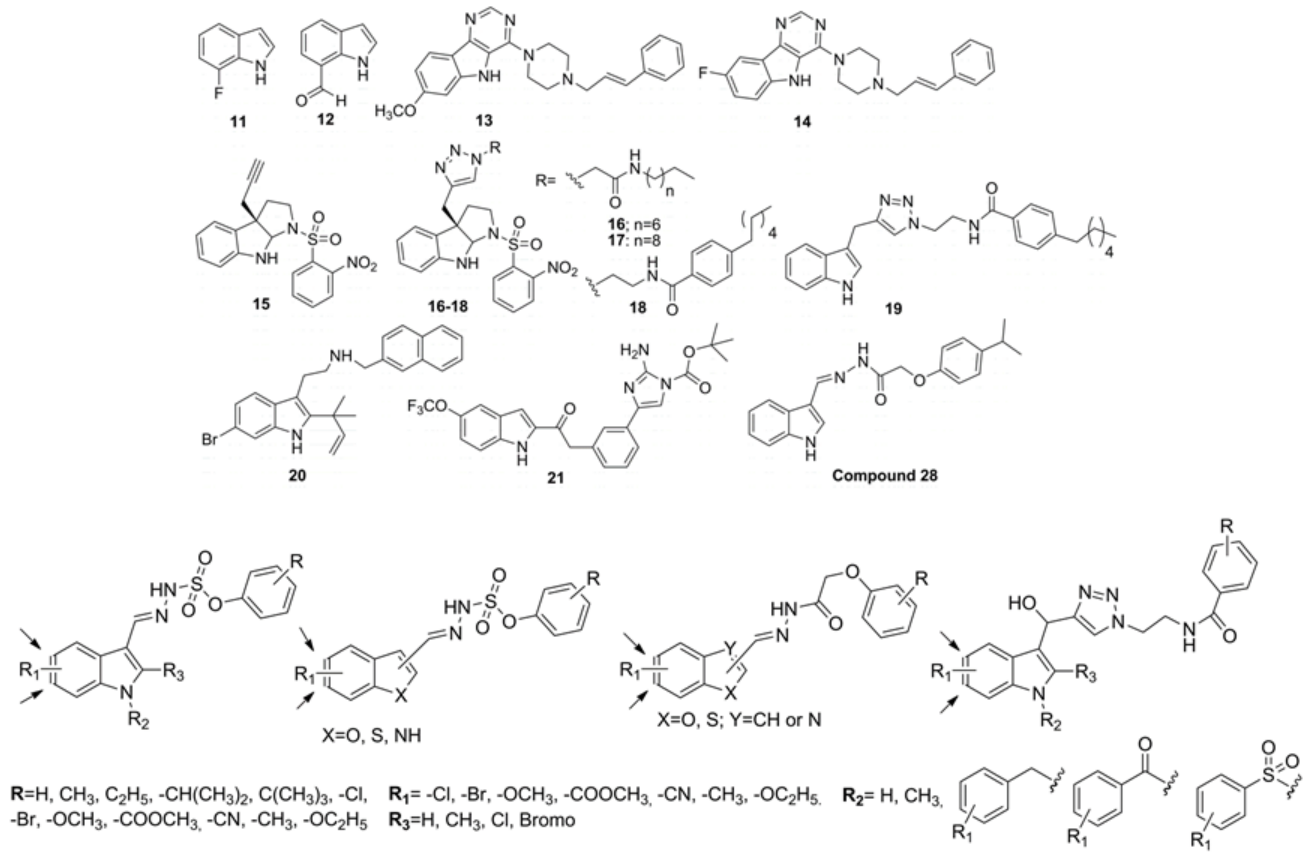

Fig. 5. Structures of biofilm-inhibitory molecules. Top: Structures of indole-based analogs found to inhibit biofilm formation in various bacterial species shown relative to that of Compound 28. Bottom: Novel structures illustrating analogs of Compound 28 predicted to have greater structural stability and potency.

KEY RESEARCH ACCOMPLISHMENTS

1. Identified one compound from the original library that we believe, with further optimization of its pharmacological properties, will ultimately prove to be therapeutically relevant.

2. Synthesized analogs of this compound that hopefully will prove to have greater stability and improved pharmacological properties without diminishing its biological properties.

3. Began to investigate novel methods for the targeting delivery of conventional antibiotics as well as small molecule inhibitors identified in our studies directly to bone.

REPORTABLE OUTCOMES

The experiments being carried out under the auspices of this project do not involve human subjects and therefore do not have reportable outcomes.

CONCLUSION

By comparison to the original 3 year timetable of this project, the experiments were delayed owing to the lack of stability of our most promising compound, thus precluding our ability to proceed to *in vivo* experiments. However, we did identify one compound of tremendous promise, the issue being that it lacked the necessary chemical stability. Thus, we shifted our efforts to build on the results of our primary screen by using this compound as a chemical starting point for the synthesis of more stable and pharmacologically active compounds. Screening these compounds will put us in a position to move forward with animal studies focusing on those that offer the greatest therapeutic promise. Thus, we are confident we will ultimately

accomplish the objectives of this project, and we are equally confident that the results we ultimately obtain will have a significant impact on the clinical approach and, more importantly, the therapeutic outcome in biofilm-associated infections arising from traumatic injury including those directly associated with military service.

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